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OF THE FIBROIN OF SILK

BY ROBERT LORIMER GRANT

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIRE-
MENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE
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SOME PRODUCTS OF PARTIAL HYDROLYSIS OF SILK FIBROIN*

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The chemistry of silk fibroin and of the various products of its hydrolysis has been the subject of extensive investigation by Abderhalden and his coworkers (1). Interest has been centered around the "silk peptone" of Abderhalden and Steinbeck (2) and the diketopiperazines, which Abderhalden believes to be primary products of the hydrolysis of silk fibroin. Uchino (3) has studied recently the products of partial degradation of fibroin by various hydrolytic agents and has interpreted his data as evidence against the existence of the diketopiperazines as primary products of hydrolysis.

The purpose of the present investigation was the study of the composition and properties of peptones prepared from silk under various conditions of hydrolysis. It was hoped that, from the products of incomplete hydrolysis of silk, it might be possible to obtain substances of widely different composition, which would be of value in the study of the problems of structure.

EXPERIMENTAL

The material used for hydrolysis was silk waste of the grade designated in commerce as No. 6 short noils,¹ containing 4.73 per

* A preliminary account of this investigation was presented before the Twenty-eighth meeting of the American Society of Biological Chemists at New York, March 28-31, 1934 (Lewis, H. B., and Grant, R. L., *J. Biol. Chem.*, **105**, lii (1934)).

¹ This product has been degummed to remove the sericin. We were unable to extract any protein by prolonged treatment with boiling water from the noils used in our experiments. We wish to express our indebtedness to the Cheney Brothers of South Manchester, Connecticut, for their courtesy in supplying the silk used in these experiments.

cent moisture, 0.50 per cent ash, and 0.33 per cent of sulfur. Other analyses of the silk are given in Table I.

Two methods were used for the determination of amino nitrogen, the gasometric method of Van Slyke and the titration method of Harris (4), a method by which it was possible to estimate carboxyl groups also. Tyrosine was determined by the modified Folin-Ciocalteu method suggested by Folin and Marenzi (5) and glycine as the ester hydrochloride.

TABLE I

Analyses of Silk Peptones Prepared by Method of Abderhalden and Steinbeck (2) but with Varying Periods of Hydrolysis

For each hydrolysis, 10 gm. of silk were used. All values are calculated on an ash-free moisture-free basis.

Preparation No.*	Yield	Total N	Amino N of total N		Tyrosine
			Van Slyke	Harris	
	gm.	per cent	per cent	per cent	per cent
Silk†		18.0			12.3
2-1	5.6	18.0	19.6	17.5	10.9
2-2	6.4	17.5	19.5	17.9	11.0
4-1	6.0	17.6	25.8	21.6	11.0
4-2	6.3	17.2	25.1	21.3	11.2
7-1	6.5	16.9	33.7	29.3	10.6
7-2	7.7	16.7	33.9	29.9	11.0
14-1	6.8	16.7	46.0	40.1	10.5
14-2	6.9	16.5	45.9	38.5	10.6
21-1		16.0	64.1	61.8	10.1

* The figures before the hyphen represent the duration in days of the hydrolysis; thus Preparations 2-1 and 2-2 are duplicate preparations which were obtained after hydrolysis at room temperature for 2 days.

† Glycine, 31.6 per cent.

In five preparations of silk peptones,² made by the method of Abderhalden and Steinbeck (2), in which hydrolysis is effected by 70 per cent sulfuric acid at room temperature for 4 days, the nitrogen content ranged from 17.0 to 17.8 per cent; free amino nitrogen (Van Slyke), from 18.9 to 25.3 per cent; tyrosine, from 9.1 to 10.3 per cent.

² We have used the term peptone for convenience in referring to the products obtained throughout with full realization that the products are probably not homogeneous and certainly not uniform.

Peptones were also prepared similarly from the hydrolysates of silk, the variable factor being the time of hydrolysis (2 to 14 days). The analyses of the peptones prepared in this series are presented in Table I. It is to be noted that as the period of hydrolysis at room temperature increased, there was a progressive decrease in the percentage of total nitrogen of the peptone and an increase in the percentage of the total nitrogen present as free amino nitrogen in the peptone. Table I also includes an analysis of a peptone prepared from a 21 day hydrolysate. This product, analyzed by the Van Slyke method, showed 64.1 per cent of the total nitrogen as free amino nitrogen. This high figure can best be explained by the presence of free amino acids precipitated by the alcohol with the peptone. However, all the free amino nitrogen values as measured by this method may be too high since Abderhalden and Van Slyke (6) have demonstrated that abnormally high results for amino nitrogen may be obtained in the Van Slyke gasometric procedure, if the free amino group is present in a glycine molecule in peptide combination with glycine or other amino acids. Since we do not know the exact composition of these peptones nor the relative proportion of the free amino groups which may be present in the glyceryl radicals, it is impossible to estimate the magnitude of any error involved in the Van Slyke determinations. That some error is introduced into the Van Slyke method by the high content of glycine of the silk peptones is indicated by the lower values for amino nitrogen, as determined by the Harris method.

The tyrosine content of the peptones prepared after varying periods of hydrolysis appeared to have no significant relation to the duration of the hydrolysis. All the values, however, were definitely lower than that of the original silk.

It appeared that the hydrolysis had progressed to the point where fractionation of the peptones by simple methods was impossible. Accordingly, an attempt was made to find conditions under which the products of hydrolysis might be separated into fractions which would vary in composition. A product was obtained after a much shorter period of hydrolysis than those previously employed, and separated into two fractions of widely different tyrosine content. Since such a preparation and fractionation has not been reported previously, the detailed procedure is given. 100 gm. of dry silk, from which all lumps had been removed by pulling apart with the hands, were packed into a compact mass

in a 2 liter flask, 200 cc. of 70 per cent sulfuric acid were added, and the flask was incubated at 30°. After 70 minutes, practically all of the silk was in solution and the brown syrup was poured into 2 liters of ice water. A small amount of a gummy precipitate was filtered off and discarded. The sulfuric acid was removed as the insoluble barium salt and the clear light yellow filtrate and washings were concentrated at 40° under diminished pressure to approximately 300 cc. After standing overnight in the refrigerator, the syrup solidified.

The grayish white solid mass was ground with water in a mortar and transferred to a beaker with 1500 cc. of water. A considerable part of the material remained undissolved and the mass appeared somewhat gelatinous. The mixture was centrifuged and the insoluble fraction (Preparation 70-A) was washed several times with water and twice with alcohol before it was removed from the centrifuge cups and placed in 95 per cent alcohol. On the following day, it was filtered off on a Buchner funnel, washed with alcohol and ether, and dried in a vacuum oven at 60° before analysis. The yield (corrected for ash and moisture) was 22.6 gm.

The supernatant fluid and all the washings from Preparation 70-A were combined and concentrated as before to 100 cc. The syrup thus obtained was poured into 10 volumes of absolute alcohol and a precipitate (Preparation 70-B) was obtained in a yield of 26.8 gm.

The less soluble fraction (Preparation 70-A) was a grayish white powder, practically insoluble in cold water and dilute acids, slightly soluble in hot water, and readily soluble in dilute alkali. When dispersed in lithium bromide solution, it dialyzed readily and completely through a collodion membrane (7). This was in contrast to the behavior of the original silk when similarly dispersed. Immunologically it was distinct from the product obtained from the original silk (7). The more soluble fraction (Preparation 70-B) was partially soluble in cold water. Both products gave brilliant pink biuret tests. Analytical data are presented in Table II.

In a second experiment, essentially the same procedure was used except that the time of hydrolysis was 65 minutes. Two similar fractions (Preparations 65-A and 65-B) were obtained. In a third experiment by a slight variation of the procedure, three

fractions were obtained. The period of hydrolysis was 70 minutes at 30°. After removal of the acid and the excess barium, the concentrated filtrate required 7 days in the refrigerator before it solidified. The insoluble fraction (Preparation 70-A-2) was separated, as already described. The filtrate after concentration to 100 cc. was allowed to stand in the refrigerator for 24 hours. A granular yellow precipitate was filtered off, washed with alcohol and ether, and dried (Preparation 70-X). The filtrate and wash-

TABLE II

Analysis of Silk Peptones Obtained after Short Periods of Hydrolysis and Fractionated As Described in Text

The yields are expressed as gm. of ash-free moisture-free product obtained from 100 gm. of silk. All chemical analyses are calculated on a moisture-free ash-free basis.

Preparation No.	Yield	Total N	Tyrosine	Glycine	Percentage equivalent of total N as			
					Amide N	Amino N (Harris)	Carboxyl (Harris)	Tyrosine N
	gm.	per cent	per cent	per cent				
70-A	22.6	18.4	19.1			3.0*	3.2	8.0
70-B	26.8	18.9	5.7			10.7†	10.7	2.3
65-A	40.3	18.0	14.9			2.9	3.6	6.4
65-B	14.0	18.5	4.1			14.1	14.4	1.7
70-A-2‡	27.4	18.4	17.9	30.5	1.9	1.7	2.7	7.5
70-X	11.8	18.8	8.4	33.0	2.4	1.8	3.0	3.4
70-B-2	10.6	18.3	2.5	31.0	4.1	17.1	19.1	1.0

* Amino nitrogen by the Van Slyke method, 3.0 per cent.

† Amino nitrogen by the Van Slyke method, 11.0 per cent.

‡ Carbon, 49.0 per cent; hydrogen, 6.3 per cent; nitrogen (Dumas), 18.3 per cent. We are indebted to Mr. N. H. Fell for these analyses.

ings from this fraction were concentrated and poured into absolute alcohol, from which the precipitate (Preparation 70-B-2) was filtered off, washed, and dried as before.

From inspection of Table II it is evident that the more insoluble fraction contained in each case more tyrosine than the original silk (12.3 per cent), while in the more soluble fractions, the tyrosine content was low, comparable to that of the usual type of proteins. Striking differences are also to be noted in the free amino and carboxyl groups. Little free amino nitrogen was present in

the less soluble fractions, indicating that relatively few peptide linkages have been broken by the acid, while in the more soluble fractions, the higher content of free amino groups indicated a considerable degree of hydrolysis.

Glycine determinations were made on the fractions of the third experiment, Preparations 70-A-2, 70-X, and 70-B-2. The results of the analyses, 30.5, 33.0, and 31.0 per cent respectively, failed to demonstrate marked variations in the glycine contents of these fractions. All the glycine values were approximately the same as that of the silk, 31.6 per cent.

It is evident that the silk has been separated into fractions of such complexity that typical biuret tests are obtainable, but of widely different tyrosine content. In the case of Preparations 70-A and 70-B, 47 per cent of the tyrosine content of the original silk was contained in the peptones isolated. Of this, approximately 74 per cent was present in the more insoluble fraction. Similar results may be calculated in the case of the other fractions. Such separations have never been obtained with silk proteins, so far as is known to us. Abderhalden (1) has isolated from products of the hydrolysis of silk, a number of amino acid anhydrides, the diketopiperazines. We have been unable to obtain evidence of the presence of these in our products, since treatment with ethyl acetate, a solvent for the common piperazines isolated from silk, did not cause the extraction of any significant amount of material from Preparations 70-A-2, 70-X, and 70-B-2.

Muldrew (8) has fractionated the products of peptic hydrolysis of casein into a primary proteose containing neither tyrosine nor tryptophane and a secondary proteose rich in both amino acids. Jones and Gersdorff (9) have similarly obtained three fractions from a peptic digestion of casein, in two of which cystine is absent. While neither these products nor our own can be considered to be definite chemical compounds rather than mixtures, by this method it has been possible to concentrate one particular amino acid in one fraction. In this connection, the comment of Hunter (8) on the work of Muldrew is of interest. "It is an obvious advantage in dealing with a highly complex combination of amino acids to be able thus to localize even two of them in one particular fragment of the whole."

Further work on the chemical and biological properties of the

products of the partial hydrolysis of the proteins of silk is in progress.

SUMMARY

Silk has been subjected to hydrolysis at 30° with 70 per cent sulfuric acid for 65 to 70 minutes and the products of hydrolysis studied. It has been possible to obtain two peptones of widely different composition. The first had a low amino nitrogen content and a content of tyrosine higher than that of the original silk. The second contained more amino nitrogen (10 to 17 per cent of the total nitrogen) and tyrosine in amounts similar to many of the more common types of proteins (2.5 to 5.7 per cent).

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